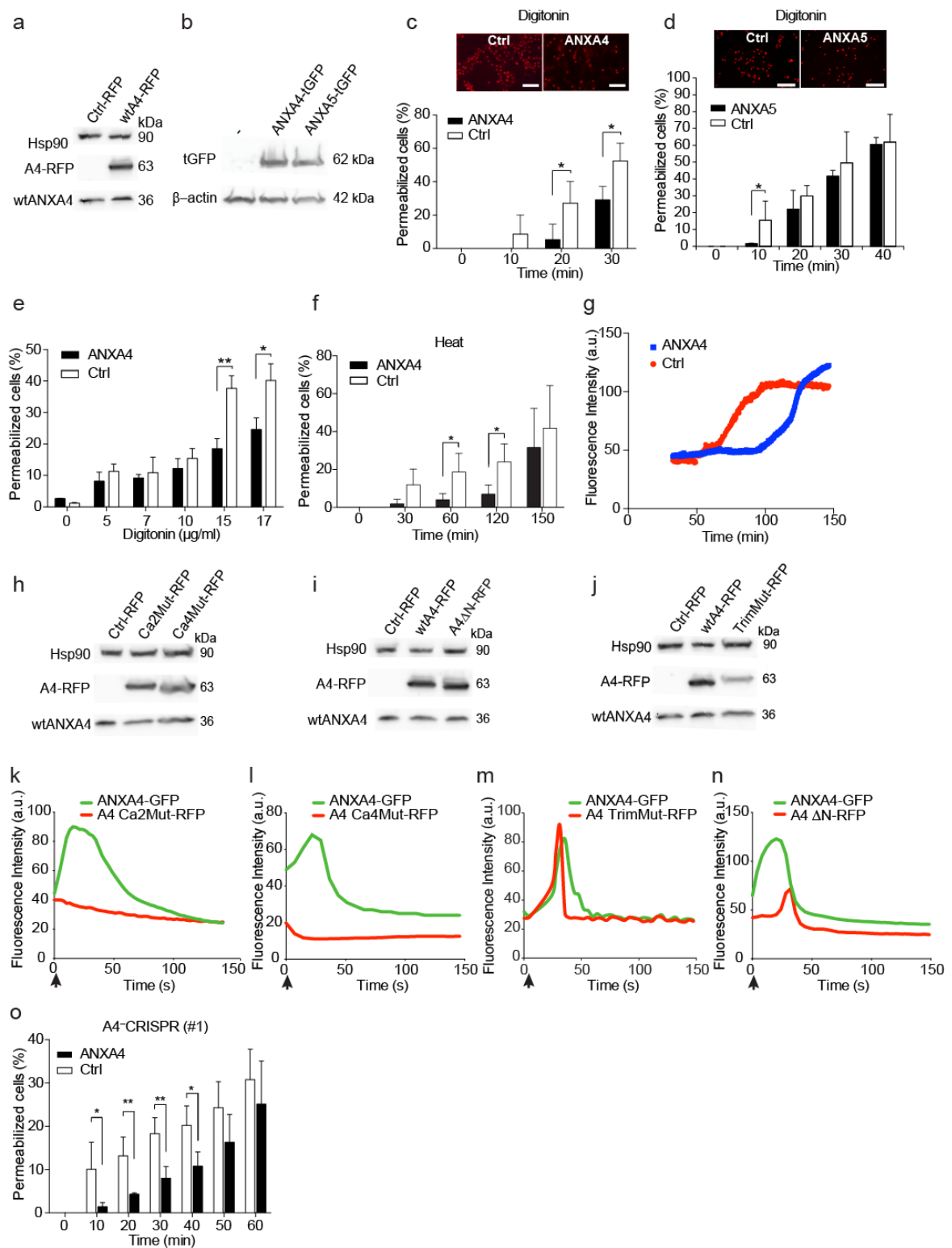
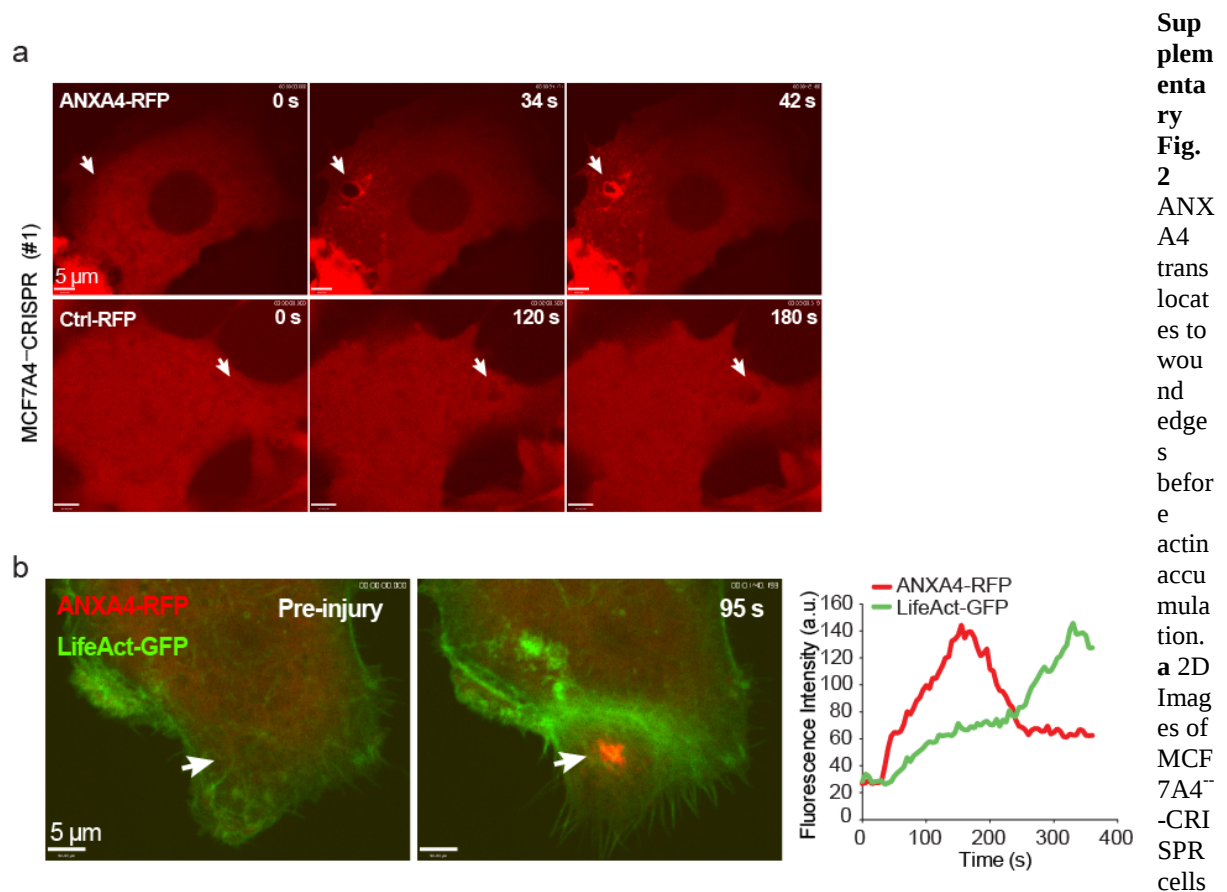


Supplementary Information

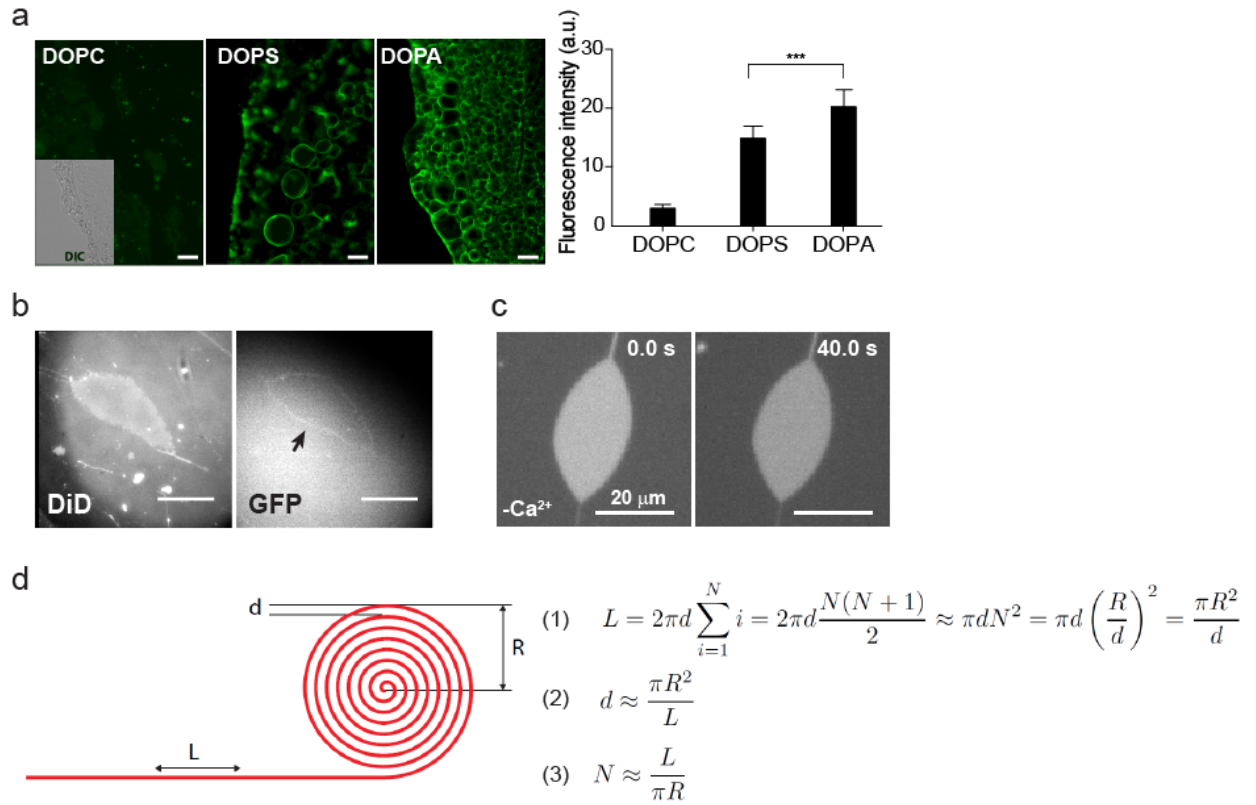


Supplementary Fig. 1 ANXA4 repairs injuries triggered by digitonin and heat shock treatment. **a** Immunoblot of lysates from MCF7 cells overexpressing wtANXA4 fused to tRFP or **b** to tGFP or ANXA5 fused to tGFP. **c**

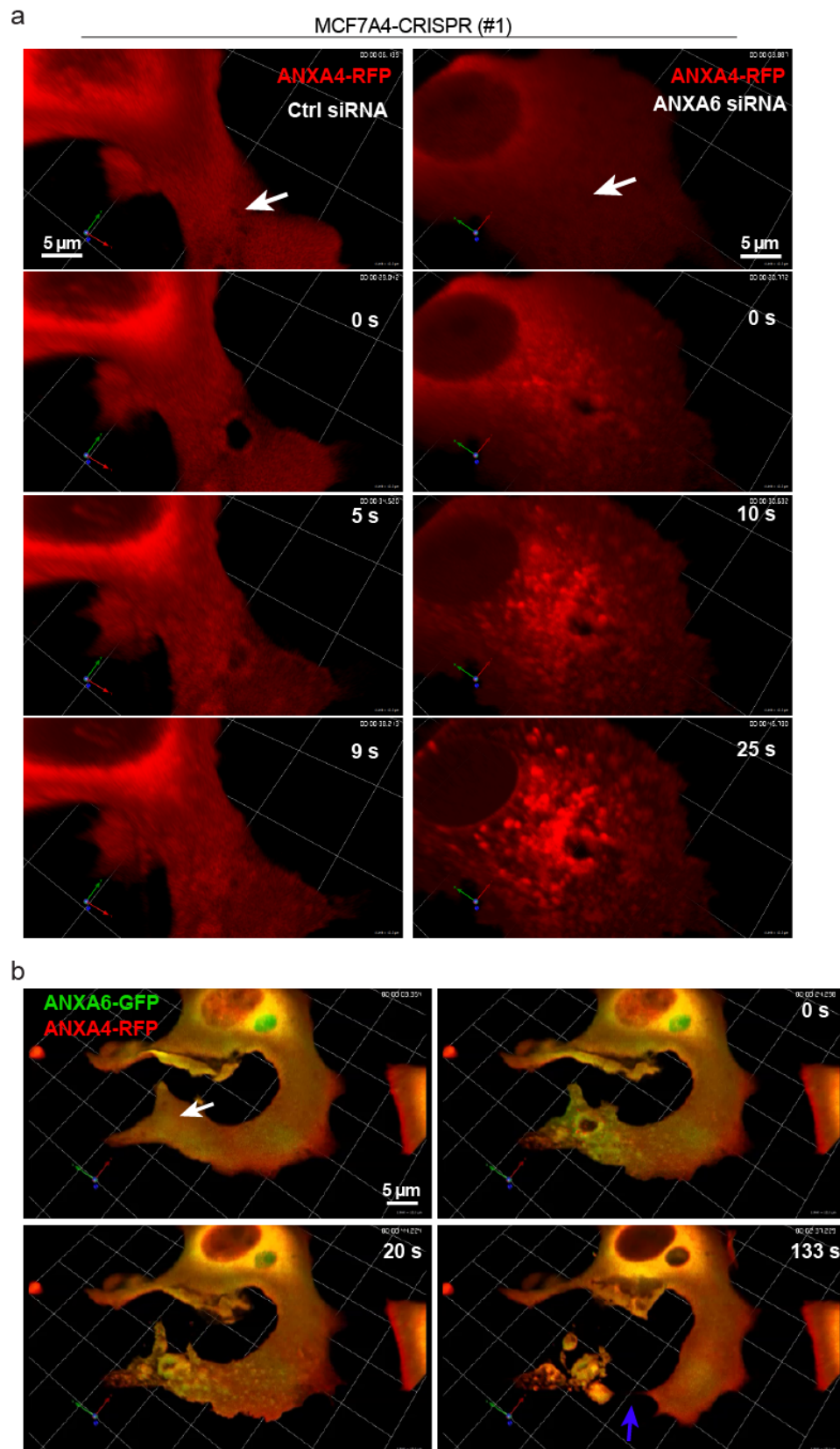
Plasma membrane integrity after treatment with digitonin (20 $\mu\text{g/mL}$) in MCF7 cells overexpressing ANXA4-RFP or **d** ANXA5-GFP as compared to Ctrl-GFP expressing cells. Permeabilized cells were imaged by live-cell microscopy using impermeable Hoechst-33258 influx as readout. Upper panel: representative images extracted from time-lapse movies showing permeabilized Hoechst-33258 positive nuclei (red pseudo-color). **e** MCF7 cells treated with increasing digitonin concentration and plasma membrane integrity measured by propidium iodide exclusion assay. **f** Quantification of 43°C heat shock induced permeabilization of MCF7 cells expressing ANXA4-RFP or Ctrl-RFP as measured by Hoechst-33258 influx. **g** Corresponding plasma membrane repair kinetics measured by FM1-43 uptake in cells exposed to 43°C heat shock in two representative cells. Quantification of permeabilized cells was performed from ≥ 3 independent experiments for each condition measuring >40 cells in each experiment. P-values based on t-test: * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$. **h** Immunoblot of lysates from MCF7 cells overexpressing Ca2Mut-RFP or Ca4Mut-RFP, **i** A4 ΔN -RFP or **j** TrimMut-RFP. **k-n** Translocation kinetics of wildtype ANXA4-GFP and different mutants upon laser injury in HeLa cells. Cells co-expressing wildtype ANXA4-GFP and **k** Ca2Mut-RFP, **l** Ca4Mut-RFP, **m** TrimMut-RFP or **n** A4 ΔN -RFP were injured and fluorescence intensity measured at the site of injury. Results are representatives of at least three experiments for each condition. **o** Membrane integrity upon 55 °C heat shock in MCF7A4⁻-CRISPR (#1) cells with introduced ANXA4-RFP or Ctrl-RFP and assayed by Hoechst-33258 exclusion as in **f**.



before and after a large laser injury (arrow) showing localization of ANXA4-RFP to the wound edge and subsequent gradual wound closure as compared to Ctrl-RFP (lower panel). **b** Sequential images of ANXA4-RFP and LifeAct-GFP (F-actin marker) behavior in MCF7 cell before and after localized laser injury (white arrow) with corresponding fluorescence plot (right).



Supplementary Fig. 3 Effect of ANXA4 on membranes. **a** Representative images of giant liposomes with different lipid composition upon addition of recombinant ANXA4-GFP protein (50 $\mu\text{g/ml}$) in the presence of Ca^{2+} (2 mM). Phosphatidylcholine (DOPC) was used as carrier lipid constituting 90% of the liposomes, while phosphatidylserine (DOPS) or phosphatidic acid (DOPA) constituted 10%. 0.1% DOPE-Atto 647N and light microscopy was used to visualize the liposomes. Scale bar, 20 μm . Quantification (right plot) of ANXA4-GFP fluorescence intensity from 10 different areas with giant liposomes. *** $P \leq 0.001$ based on students t-test. **b** Membrane patch stained with DiD after addition of ANXA4-GFP protein showing initial binding of ANXA4-GFP to the edges of the membrane patch. **c** Membrane patch incubated with wtANXA4 in the absence of Ca^{2+} . **d** Model for rolling of planar membranes induced by binding of a curvature-inducing protein. A certain region (patch) of the membrane has initially a length L measured in the direction perpendicular to the rolling axis. After completion of rolling, a cylindrical roll with radius R has formed. The period length between layers inside the roll is d . The model is simplified by approximating the roll as concentric circles, which is a fair approximation when the number of turns N is large. Equations (1-3) provide relationships between N , d , R and L assuming that $N \gg 1$ and that the radius of the roll can be written as $R = Nd$.

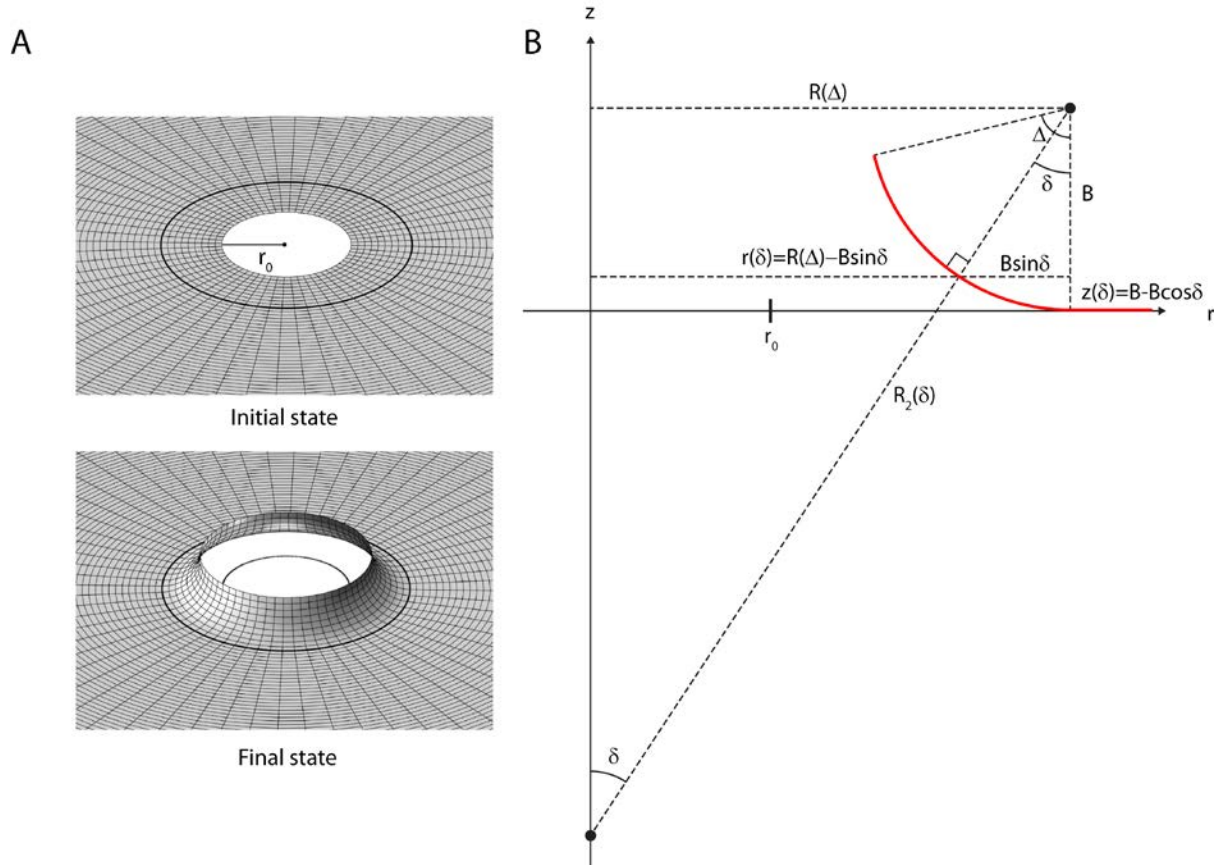


Supplementary Fig. 4 ANXA6 is needed for wound closure. **a** Sequential 3D images of MCF7A4⁻-CRISPR cells expressing wildtype ANXA4-RFP, and treated with Ctrl or ANXA6 siRNA and exposed to laser injury (white arrow indicate injury site). Note: ANXA6 siRNA treated cell is eventually dying from the injury. **b** Representative sequential images of wound healing involving two mechanisms: first ANXA4-RFP/ANXA6-GFP translocation to wound edges initiates local wound closure. Secondly, excision of the damaged part of the membrane (blue arrow). Also see Supplementary Movie 3.

Supplementary note 1

Model for neck formation at a membrane hole

Definitions and assumptions



Supplementary Fig. 5 Schematic of the model for neck formation. **A** Initial and final (curved) state of the membrane hole. The area inside the solid circle contributes to the change in curvature elastic energy. **B** Profile of the neck and definitions of quantities used in the calculation.

We consider curvature close to a hole in a membrane with rotational axial symmetry. In the initial state, the membrane has a circular hole with radius r_0 and is planar. Upon binding of a protein (e.g. annexins) to the membrane surface the effect of the protein is modeled as the induction of a spontaneous curvature (c_0) of the combined membrane/protein sheet. This is assumed to result in a partial or complete rolling around the edge of the hole, leading to a neck-like geometry. The curvature radius of the roll is B and the rolling angle (denoted *neck angle*) is Δ . The circular profile of the neck is assumed based on the experimental results for membrane rolling in supported membrane patches. We also make the simplifying assumption that $B \cdot c_0 = 1$, i.e. that the in-plane radius of curvature is equal to the radius of spontaneous curvature. The difference in elastic energy $\Delta H(\Delta)$ between the final (curved) state and the initial state is calculated. The energetically favored final state corresponds to the rolling angle $\Delta_{\min}$ that gives a minimum value of ΔH .

Description of model

The energy in the initial state is:

$$H_0 = H_{0\lambda} + H_{0A} \quad (1)$$

where $H_{0\lambda}$ is the energy contribution from line tension at the hole edge and H_{0A} is the

curvature elastic energy of the area A in the initial (planar) state.

The energy in the final state is:

$$H = H_\lambda + H_M + H_G \quad (2)$$

where H_λ is the energy contribution from line tension at the hole edge, H_M is the mean curvature elastic energy and H_G is the gaussian curvature elastic energy, both for the area A . The total change in energy upon formation of the neck is:

$$\Delta H = H - H_0 = H_\lambda - H_{0\lambda} + H_M + H_G - H_{0A} \quad (3)$$

We therefore need to compute the terms in equation (3). The energy of the membrane edge is modeled by a line tension λ . The general expression for the curvature elastic energy H_{curve} of an area A is, according to Helfrich[1]:

$$H_{\text{curve}} = H_M + H_G = \int_A \left[\frac{1}{2} k_c (\bar{c} - c_0)^2 + k_G \bar{c}_G \right] dA \quad (4)$$

where k_c is the *mean curvature elastic modulus* [J], k_G is the *gaussian curvature elastic modulus* [J] and c_0 is the spontaneous curvature [m^{-1}]. The local curvature of the membrane is described by the two principal radii of curvature, R_1 and R_2 . The mean curvature is defined as: $\bar{c} = \frac{1}{R_1} + \frac{1}{R_2}$ and the gaussian curvature as: $\bar{c}_G = \frac{1}{R_1} \frac{1}{R_2}$.

Area A of the neck region

The area A of the curved neck region is marked by the black circle in Supplementary Figure 5A. As we only consider bending deformations in this model, A must remain constant between the initial and the final states, so that the energy contribution from an area change is zero. This requirement determines the value of $R(\Delta)$, that is the r-coordinate of the center of the circle defining the in-plane curvature. The area in the initial (flat) state is:

$$A_{\text{initial}} = \pi R^2 - \pi r_0^2 \quad (5)$$

The area in the final (curved) state is:

$$\begin{aligned} A_{\text{final}} &= \int_A dA = \int_0^\Delta 2\pi r B d\delta = \int_0^\Delta 2\pi (R - B \sin \delta) B d\delta \\ &= 2\pi B (R\Delta + B \cos \Delta - B) \end{aligned} \quad (6)$$

The requirement $A_{\text{final}} = A_{\text{initial}}$ gives the following expression for R :

$$\frac{R}{B} = \Delta + \sqrt{\Delta^2 + \left(\frac{r_0}{B}\right)^2 + 2(\cos \Delta - 1)} \quad (7)$$

or when $B \cdot c_0 = 1$:

$$Rc_0 = \Delta + \sqrt{\Delta^2 + (r_0 c_0)^2 + 2(\cos \Delta - 1)} \quad (8)$$

Radii of curvature R_1 , R_2

In order to compute the mean and gaussian curvature elastic energy in the final state we need to know the radii of curvature R_1 and R_2 at any point on the curved neck. Referring to Supplementary Figure 5B we define the in-plane radius of curvature as the constant $R_1 = B$. The out-of-plane radius of curvature is found according to the lower triangle in Supplementary Figure 5B:

$$\frac{1}{R_2} = -\frac{\sin \delta}{r(\delta)} \quad (9)$$

The angle $\delta \in [0, \Delta]$ describes any point on the cylindrically symmetric neck and $r(\delta) = R - B \sin \delta$. The minus in equation (9) is due to the opposite curvature with respect to R_1 . Finally, we get for the mean and gaussian curvature:

$$\bar{c} = \frac{1}{R_1} + \frac{1}{R_2} = \frac{1}{B} - \frac{\sin \delta}{r} \quad \bar{c}_G = \frac{1}{R_1} \frac{1}{R_2} = -\frac{1}{B} \frac{\sin \delta}{r} \quad (10)$$

Change in line tension energy: $H_\lambda - H_{0\lambda}$

The line tension energy increases due to expansion of the hole. The change is:

$$H_\lambda - H_{0\lambda} = 2\pi\lambda r(\Delta) - 2\pi\lambda r_0 \quad (11)$$

$$= 2\pi\lambda B \left(\frac{R}{B} - \frac{r_0}{B} - \sin \Delta \right) \quad (12)$$

$$= 2\pi \frac{\lambda}{c_0} (Rc_0 - r_0c_0 - \sin \Delta) \quad (13)$$

where λ is the line tension for a free membrane edge. Equation (13) is valid for $B \cdot c_0 = 1$.

Elastic bending energy in the initial state: H_{0A}

In the initial state, the membrane is flat and $R_1 = R_2 = \infty$. According to equation (4), the elastic bending energy in the initial state becomes:

$$H_{0A} = \frac{1}{2} k_c c_0^2 A \quad (14)$$

Inserting the area from equation (5) gives:

$$H_{0A} = \frac{\pi}{2} k_c c_0^2 (R^2 - r_0^2) \quad (15)$$

Gaussian curvature elastic energy in the final state: H_G

The gaussian curvature elastic energy in the final state is:

$$H_G = \int_A k_G \bar{c}_G dA \quad (16)$$

$$= \int_A k_G \frac{1}{R_1} \frac{1}{R_2} dA \quad (17)$$

$$= - \int_0^\Delta k_G \frac{1}{B} \frac{\sin \delta}{r} 2\pi r B d\delta \quad (18)$$

$$= 2\pi k_G (\cos \Delta - 1) \quad (19)$$

Note that this result is independent of r_0 and c_0 .

Mean curvature elastic energy in the final state: H_M

The mean curvature elastic energy in the final state is:

$$H_M = \int_A \frac{1}{2} k_c (\bar{c} - c_0)^2 dA \quad (20)$$

$$= \int_A \frac{1}{2} k_c \left(\frac{1}{B} - \frac{\sin \delta}{r} - c_0 \right)^2 2\pi r B d\delta \quad (21)$$

With the assumption $B \cdot c_0 = 1$ this becomes:

$$H_M = \pi k_c \int_0^\Delta \frac{\sin^2 \delta}{r c_0} d\delta \quad (22)$$

$$= \pi k_c \int_0^\Delta \frac{\sin^2 \delta}{R c_0 - \sin \delta} d\delta \quad (23)$$

The integral in equation (23) is evaluated numerically for practical reasons.

Supplementary note 2

Results

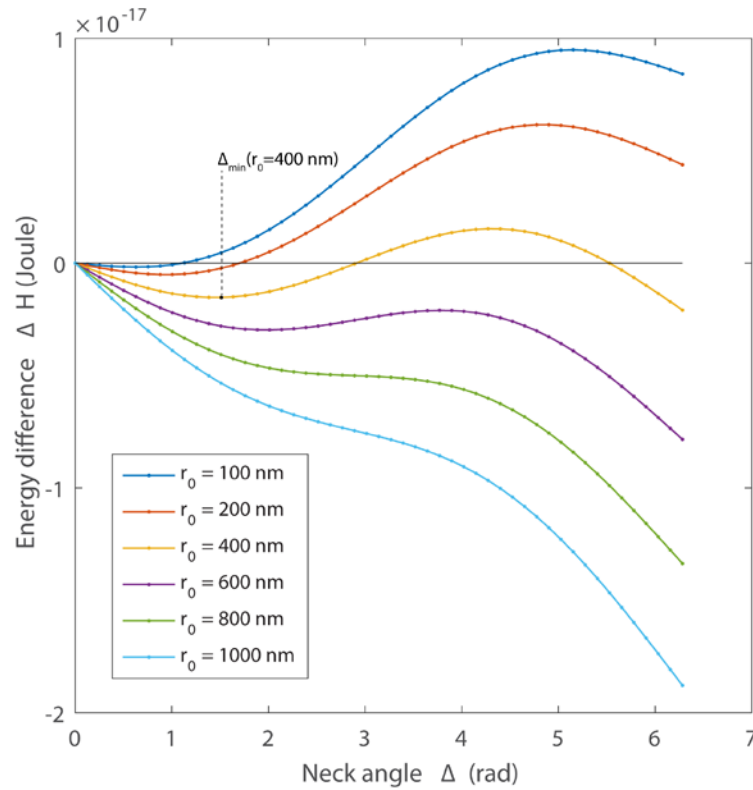
To evaluate equation (3) we need values for the parameters: λ , k_c , k_G and c_0 . Precise values corresponding to our experimental system (POPC, POPS 9:1 or cellular membranes) are not available in the literature and we therefore use the following order-of-magnitude estimates based on similar lipid systems:

$\lambda = 10$ pN. Reference [2] reports the values 6.9 pN and 20.7 pN for DOPC from the companies Sigma and Avanti respectively.

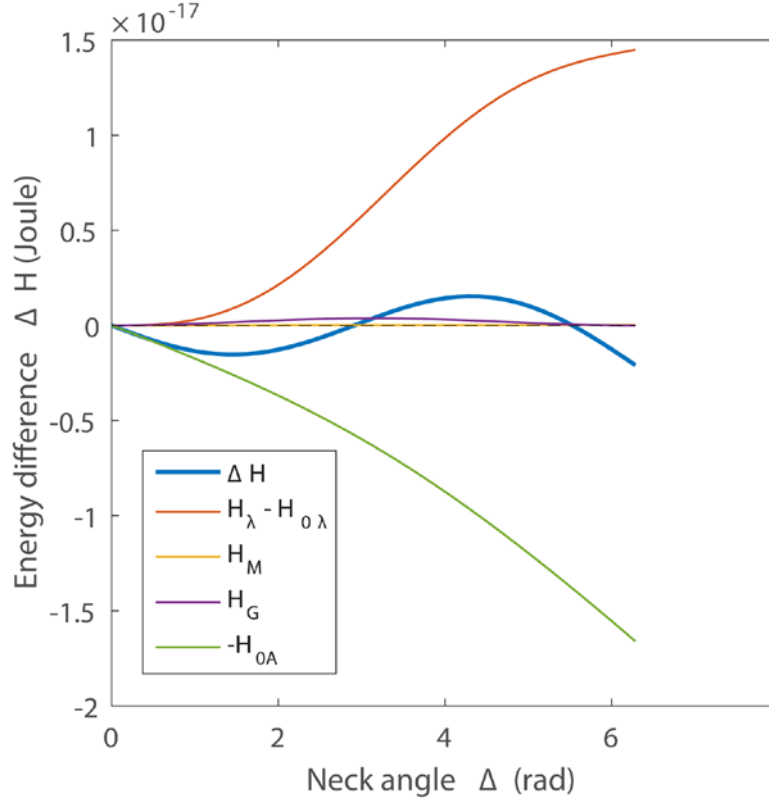
$k_c = 4.0 \cdot 10^{-20}$ J. From Marsh[3], table II.10.4.1, page 474. Reported values for POPC are from 2.5 - $8.5 \cdot 10^{-20}$ J.

$k_G = -3.0 \cdot 10^{-20}$ J. From Marsh[3], table II.10.4.2, page 477. Based on monolayer results showing $k_G/k_c \approx -0.75$

$c_0 = 0.033$ nm⁻¹. Based on computational modeling of Shiga toxin by Pezeshkian et. al. [4].



Supplementary Fig. 6 Energy difference ΔH in equation (3) as a function of the neck angle Δ for varying values of the initial hole size r_0 . For values of r_0 below a critical hole radius r_0^* there is a minimum in ΔH corresponding to an energetically favored neck angle $\Delta_{\min}$. In the plot $r_0^* \approx 800$ nm. For $r_0 > r_0^*$, ΔH decreases monotonically with Δ meaning that rolling is favored.



Supplementary Fig. 7 Contributions to the energy change ΔH from the terms in equation (3) for $r_0 = 400$ nm. Note that the increase in line tension energy ($H_\lambda - H_{0\lambda}$) and the curvature energy in the initial state ($-H_{0A}$) dominates.

To obtain an approximate expression for the critical hole radius r_0^* we neglect the terms (H_M) and (H_G) in equation (3) and expand in Δ . This gives:

$$\Delta H \simeq 2\pi \frac{\lambda}{c_0} (\Delta - \sin \Delta) - \pi k_c r_0 c_0 \Delta \quad (24)$$

We find Δ_{min} by differentiation:

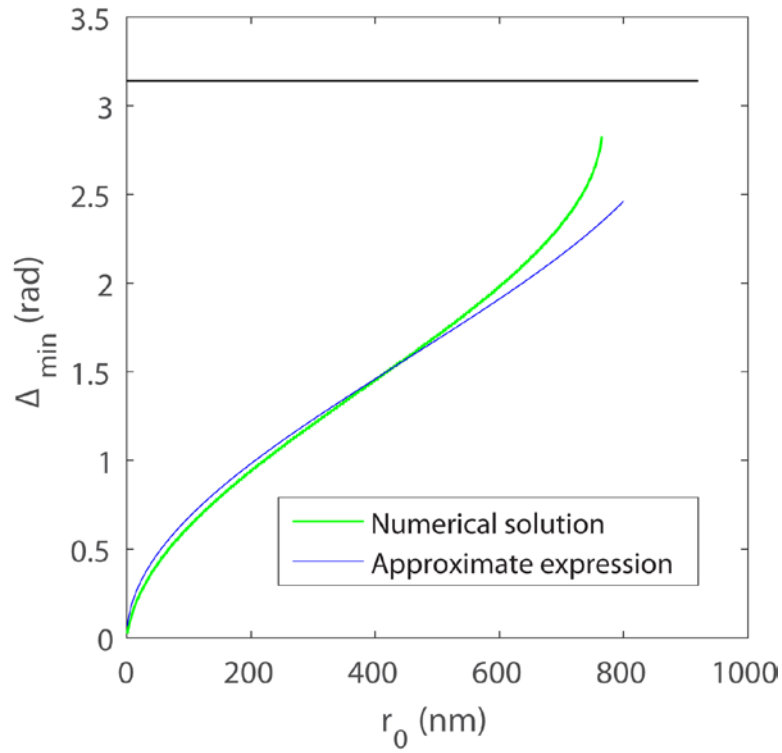
$$\left. \frac{\partial \Delta H}{\partial \Delta} \right|_{\Delta_{min}} = 0 \quad (25)$$

$$\Delta_{min} \simeq \cos^{-1} \left[1 - \frac{k_c c_0^2 r_0}{2\lambda} \right] \quad (26)$$

This has a solution only if $r_0 > 0$ and if:

$$r_0 < \frac{4\lambda}{k_c c_0^2} \simeq r_0^* \quad (27)$$

-providing an approximate value of the critical hole radius r_0^* . Equation (26) is in Supplementary Fig. 8 compared to the corresponding value determined numerically.



Supplementary Fig. 8 Variation in the energetically favored neck angle Δ_{min} with initial hole radius r_0 . Green line is determined numerically from equation (3). Blue line is the approximate solution from equation (26).

References

- [1] Helfrich, W. Elastic properties of lipid bilayers: theory and possible experiments. Z. Naturforsch. 28, 693-703 (1973).
- [2] Karatekin, E. et al. Cascades of transient pores in giant vesicles: line tension and transport. Biophys. J. 84, 1734-1749 (2003).
- [3] Marsh, D. Handbook of lipid bilayers (CRC press, 2013).
- [4] Pezeshkian, W. et al. Membrane invagination induced by shiga toxin B-subunit: from molecular structure to tube formation. Soft Matter 12, 5164-5171 (2016).